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Type I interferon responses of common carp strains with different levels of resistance to koi herpes virus disease during infection with CyHV-3 or SVCV.

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Highlights

1. Differences in mortality rates during SVCV and CyHV-3 infections were recorded in carp strains.
2. The higher resistance of the Rop strain was related to lower virus load and replication.
3. The magnitude of type I IFN response was not positively correlated with survival.
4. CyHV-3 has an ability to limit IFN response induced by sensing viral DNA by cells.

Keywords: *cyprinid herpesvirus 3*, *spring viremia of carp*, CyHV-3, SVCV, common carp, *Cyprinus carpio*, type I interferon, resistance

Abstract

Carp from breeding strains with different genetic background present diverse levels of resistance to viral pathogens. Carp strains of Asian origin, currently being treated as *Cyprinus rubrofuscus* L., especially Amur wild carp (AS), were proven to be more resistant to koi herpesvirus disease (KHVD; caused by *cyprinid herpesvirus 3*, CyHV-3) than strains originating from Europe and belonging to *Cyprinus carpio* L., like the Prerov scale carp (PS) or koi carp from a breed in the Czech Republic. We hypothesised that it can be associated with a higher magnitude of type I interferon (IFN) response as a first line of innate defence mechanisms against viral infections. To evaluate this hypothesis, four strains of common carp (AS, Rop, PS and koi) were challenged using two viral infection models: Rhabdovirus SVCV (*spring viremia of carp virus*) and alloherpesvirus CyHV-3. ~~Infection experiments confirmed significant differences in mortality between the analysed carp strains.~~ The infection with SVCV induced a low mortality rate and the most resistant was the Rop strain (no mortalities), whereas the PS strain was the most susceptible (survival rate of 78%). During CyHV-3 infection, Rop and AS strains performed better (survival rates of 78% and 53%, respectively) than PS and koi strains (survival rates of 35% and 10%, respectively). The evaluation of virus loads and virus replication showed significant differences between the carp strains, which correlated with the mortality rate. The evaluation of type I IFN responses showed that there were fundamental differences between the virus infection models. While responses to the SVCV were high, the CyHV-3 generally induced low responses. Furthermore, the results demonstrated that the magnitude of type I IFN responses did not correlate with a higher resistance in infected carp. In the case of a CyHV-3 infection, reduced type I IFN responses could be related to the potential ability of the virus to interfere with cellular sensing of foreign nucleic acids. Taken together, the results broaden our understanding of how common carp from different genetic lines interact with various viral pathogens.

1. Introduction

Common carp presently refers to two species: (i) *Cyprinus carpio* L., comprising the European common carp strains, and (ii) *Cyprinus rubrofuscus* L., also known as Asian carp [1]. In Europe, several distinctive carp strains have been developed and are bred in several live gene banks kept in fisheries institutes in the Czech Republic, Hungary and Poland [2]. Generally, these strains showed rather low to moderate resistance to *cyprinid herpesvirus 3* (CyHV-3) infection and the development of koi herpesvirus disease (KHVD), which currently poses a major threat to carp aquaculture worldwide [3-6]. Contrary to this, the Asian carp strains, like the Amur wild carp, have a much higher CyHV-3 infection survivability. Interestingly, the Ropsha strain (a crossbreed between *C. carpio* and *C. rubrofuscus*), which was historically developed by crossing Amur wild carp and common carp from Central-Eastern Europe, possesses a similar survival rate to Amur wild carp [4, 6-8]. Independent of this, the coloured variety of common carp (also known as koi carp) developed from both European and Asian carp strains has a low survivability of a CyHV-3 infection [6]. In case of infection with the *spring viremia of carp virus* (SVCV), differences in resistance among carp species have not been extensively studied. Kirpichnikov at al. [9] demonstrated that the Krasnodar common carp strain (which originated from the Ropsha carp strain) showed the highest resistance to a SVCV infection when compared to other strains [9].

Differences in the outcome of a disease can be related to several factors: The virus lacks the ability to replicate in resistant animals; for example, due to an incompatibility of the virus to the cellular receptor typically used by the virus to enter the cells. ~~This was the proposed mechanism in the case of a viral haemorrhagic septicemia virus (VHSV) resistant clonal strain of rainbow trout (Oncorhynchus mykiss). Likewise, in Atlantic salmon (Salmo salar), quantitative trait loci for infectious pancreatic necrosis virus (IPNV) resistance were shown to be located in~~

~~the genome region encoding epithelial cadherin (cdh1-1 and cdh1-2), and a single nucleotide polymorphism in cdh1-1 was associated with resistance, most likely by preventing the virus from attaching to and internalising into cells.~~ An increased survivability of fish can also be related to a more efficient immune response which possesses the ability to stop the development of the infection. One of the immune processes which is capable of this kind of impact is, for example, the antiviral response triggered by the induction of type I interferons, which are considered to be the first line of innate defence against viral infections. Multiple studies, performed *in vitro* or *in vivo*, showed that the stimulation of type I IFN production or the application of recombinant interferon has a protective effect against many different fish viruses [10-12]. Furthermore, it was shown that a strong type I IFN response in cells of a clonal line of rainbow trout (*Oncorhynchus mykiss*) was the underlining factor for the resistance of fish from this particular line against VHSV and for protecting theses fish from mortality [13]. Also, in the context of immune responses of fish to viruses with more complex genomes, like Alloherpesviruses, the magnitude of type I IFN responses was proven to be crucial for increased resistance to virus induced mortality. This was shown in very recent studies performed on infections of Prussian (gibel) carp (*Carassius gibelio*) with *Carassius auratus herpesvirus* (CaHV) [14].

In common carp type I IFN responses were earlier studied in the context of its influence on the survival of carp from a CyHV-3 infection [15, 16]. Despite the fact that the development of a CyHV-3 infection *in vitro* could be delayed by the induction of type I IFNs [17], it was shown that in European common carp lines/strains, the type I IFN response was not correlated with the differences in the resistance to CyHV-3 *in vivo* [16]. Furthermore, the results from the infection experiments seem to confirm previous *in vitro* observations that CyHV-3 is capable of mitigating the activation of the type I IFN system [16, 17]. In contrast to CyHV-3, SVCV has a

much lower ability to modulate type I IFN responses *in vitro* and induces a very high type I IFN immune response [17].

In the current research, different genetic strains of carp were selected on the basis of previous observations on the susceptibility of these strains to an infection with CyHV-3 [6]. In these experiments, lines or crossbreeds derived from carp from the Amur basin (in particular, AS and Rop carp) showed a remarkable resistance to this virus, while carp of European origin or koi experienced high morbidity and mortality [6]. The aim of the current study was to elucidate the nature of the resistance of the common carp strains to infections with CyHV-3 and SVCV by monitoring the development of the infection in four tissues and subsequently measuring type I IFN responses. Further *in vitro* studies were performed to better characterise anti-IFN actions of CyHV-3.

2. Material and Methods

2.1. Naive common carp and koi

Naive individuals from the Amur wild carp (AS), Ropsha carp (Rop), Prerov scaly carp (PS) and koi strains were obtained as five months-old fingerlings from the University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, located in Vodnany, the Czech Republic. The fish were full siblings of a stock which had been used in a previous infection experiment with the carp edema virus (CEV) [18]. Briefly: Experimental stocks of carp were obtained from artificial reproduction by means of a protocol established by Kocour et al. [19] using a full-factorial mating scheme of three females with three males per strain. The stocks were kept in a closed recirculation system supplied with tap water from their egg stage onwards. After hatching, the fish were transferred to and raised in the separate recirculation systems filled with tap water at 22 °C and fed a commercial carp feed (Aller, Denmark) at 1% of body weight per day.

For the infection experiments, the carp were transported to the National Veterinary Research Institute in Pulawy, Poland at a mean weight of 10.3 ± 8.2 g and placed at 18 °C in a flow through system two weeks prior to the infection experiment. Just after transport, the fish were marked by means of fin clipping in order to allow identification of the individual strains and then mixed in one tank. All carp strains were confirmed to be free of DNA or RNA specific for CyHV-3, SVCV, CEV and a yet unclassified RNA virus possessing characteristics of Arenavirus, Orthovirus and Paramyxovirus [20]. Gill and kidney tissues of carp (n=5 per strain) were examined for the presence of these viruses by means of qPCR or RT-qPCR as described earlier [18]. The fish were also inspected and confirmed to be free of ectoparasites by means of fresh smears from the skin and gill surfaces which were examined with a light microscope.

2.2. Infection experiments

For each of the infections, the fish were divided into six 200 L tanks. Four tanks were used for mortality analyses, including three tanks with infected fish and one tank with non-infected control fish. Two additional tanks were used for the sampling experiment, including one tank with infected fish and one tank with non-infected control fish. Before experimental infection, the fish were acclimatised to the water temperature in the infection tanks by changing the water temperature from 18 to 12 °C by 1 °C per day for the SVCV infection and 18 from to 21 or 23 °C by 1 °C per day for the CyHV-3 infection. ~~The mortality rate was observed daily for 26 days during CyHV-3 infection and 28 days during SVCV infection.~~ All animal experiments were performed in accordance with national and international regulations for experimentation with animals and under approval of the Local Ethical Committee in Lublin, Poland.

The SVCV infection was performed at 12 °C by exposing healthy SPF carp in small 20 L tanks to tissue culture derived virus for 45 min by immersion under constant aeration. The water of these tanks contained 1×10^7 $TCID_{50}$ mL^{-1} of the SVCV isolate 56-70, which was used earlier by Adamek et al. [17, 21]. After infection, the fish were returned to the 200 L tank and kept at a water temperature of 12 ± 1 °C. The same procedure was used for the mock-infected control fish, whereby the virus suspension was replaced by uninfected cell culture medium. The mortality was monitored 3 times per day for 28 days and dead fish were put to separate plastic bags and frozen in -20°C. For expression studies samples of gill, skin, kidney and head kidney (n = 5 fish per time point) were collected into RNA*later* at 12 h, 36 h, 96 h 144 h and 336 h post infection and stored at -80°C until further analyses.

For CyHV-3 infection, healthy SPF individuals from all four genetic strains were exposed to CyHV-3 of a Polish isolate derived from tissue culture at a final concentration of 2×10^3 $TCID_{50}$ mL^{-1}. The bath infection was performed in small tanks with 20 L water at 22°C for 45

166 min by immersion under constant aeration [5]. The mock-infected control was also included in
167 this experiment. After infection, the fish were returned to the 200 L tanks and kept at a water
168 temperature of 23 ± 1 °C for mortality groups and at 21 ± 1 °C for the tissue collection group. For
169 the latter group, a lower temperature was selected in order to prevent a too early onset of
170 mortality which could interfere with the tissue collection. The mortality was monitored 3 times
171 per day for 26 days and dead fish were put to separate plastic bags and frozen in -20°C. For
172 expression studies samples of gill, skin, kidney and head kidney (n = 5 fish per time point) were
173 collected into RNA*later* at 12 h, 36 h, 96 h 144 h and 336 h post infection and stored at -80°C
174 until further analyses.
175
176 **2.3. Preparation of primary fin cell cultures and subsequent infection with CyHV-3**
177 Primary fin cell cultures were established as described earlier [18, 22]. Cultures were
178 created from n = 3 individuals per strain. Fins were cut into small pieces (<10 mm^2) and placed
179 individually into the wells of 24 well tissue culture plates. 1 mL of culture medium containing
180 Medium 199 (Sigma) supplemented with 20% FBS (Sigma), 10 IU mL^{-1} penicillin, 100 mg mL^{1}
181 streptomycin, 100 mg mL^{-1} gentamycin and 1 mg mL^{-1} amphotericin B (Sigma) was added to
182 each well. Primary fin cultures were incubated at 25 °C in a humidified atmosphere containing
183 2% CO_2. After 96 hrs, cultures reaching > 50% confluence were selected for an infection with
184 CyHV-3 (Taiwan isolate, KHV-T).
185 From each fish, primary fin cultures were either infected with CyHV-3 (1 x 10^4 $TCID_{50}$
186 mL^{-1}) or mock infected (each variant in duplicate). The cultures were incubated for 48 h at 25°C
187 in a humidified incubator containing 2% CO_2. After 48 h, the medium was removed from all
188 cultures and the cells lysed in 1 mL of Tri-Reagent (Sigma) before being transferred to 1.5 mL
189 reaction tubes and stored at - 80 °C until RNA isolation.

190

191 **2.4. Effect of a stimulation of KFC cells with Z-DNA, B-DNA and dsRNA on CyHV-3**
192 **replication.**

193 Koi fin cells (KFC), a permanent fibroblast-like cell culture [23] were cultured in
194 minimum essential medium (MEM) with Earle's salts (Sigma) supplemented with NEAA
195 (Sigma), 10% FBS (Sigma, Germany), 10 IU mL $^{-1}$ penicillin and 100 mg mL^{-1} streptomycin
196 (Sigma). Cultures were incubated at 25 °C in a humidified atmosphere containing 2% CO_2. The
197 KFC cells were plated in eight 12-well plates and grown for 24h to 95% confluency.
198 Subsequently, 48 wells were infected with medium containing CyHV-3 virus (Taiwan isolate,
199 KHV-T) in a concentration allowing an infection with a multiplicity of infection (MOI) of ten, or
200 48 wells were mock infected with culture medium containing no virus. After 30 min at 25 °C, the
201 cells were washed twice with the culture medium. After this, both cells (CyHV-3 infected and
202 mock infected) were transfected with 1 µg mL^{-1} of polyinosinic polycytidylic acid (poly IC,
203 synthetic double stranded RNA; Invivogen), 1 µg mL^{-1} poly(deoxyadenylic-deoxythymidylic)
204 acid sodium salt (synthetic double stranded B-DNA; Invivogen) or 1 µg mL^{-1}
205 poly(deoxyguanylic-deoxycytidylic) acid sodium salt (synthetic double stranded Z-DNA;
206 Invivogen) using the LyoVec reagent (Invivogen) in accordance with the manufacturer's
207 instructions and with medium without FCS. The non-infected and infected controls were treated
208 only with medium containing LyoVec. Cells from n = 3 were collected 2h post transfection. This
209 was followed by a collection of samples after 8h, 36h and 96h. Collected cells were lysed in 1 mL
210 Tri-Reagent (Sigma) before being transferred to 1.5 mL reaction tubes and stored at - 80 °C until
211 RNA extraction.

212

213 **2.5. DNA extraction**

214 DNA was extracted from ~ 15 mg of tissue after mechanical lysis in a QIAgen Tissuelyser
215 II (Qiagen), using the QIAamp DNA Mini Kit (Qiagen) in accordance with the manufacturer's
216 instructions. After isolation, the samples were diluted to 50 ng μl^{-1} and stored at −80 °C.
217
218 **2.6. RNA extraction and cDNA synthesis**
219 The total RNA was extracted using Tri-Reagent (Sigma) in accordance with the
220 manufacturer's instructions. Any remaining genomic DNA was digested with 2 U of DNase I
221 (Thermo Fisher Scientific) in accordance with the standard protocol. Synthesis of cDNA was
222 performed from 300 ng of the total RNA using the Maxima™ First Strand cDNA Synthesis Kit
223 (Thermo Fisher Scientific). A non-reverse transcriptase control was included in the analysis of
224 each sample. cDNA samples were diluted 1:20 with nuclease-free water (Thermo Fisher
225 Scientific) prior to RT-qPCR analysis.
226
227 **2.7. qPCR/RT-qPCR**
228 CyHV-3 ORF55 DNA detection and quantification were performed using a probe based
229 real-time qPCR (TaqMan) modified from Gilad et al [24] as described earlier by Adamek et al
230 [22]. The results are presented as the total number of virus genome copies per 250 ng of DNA.
231 For quantification of viral (SVSV G, CyHV-3 ORF72) and host mRNA (*ifn a2, vig1, 40s*)
232 a SYBR Green based RT-qPCR was used. Reactions were performed in duplicate using the
233 Maxima SYBR Green 2× mastermix (Thermo Fisher Scientific) in a Stratagene Mx3005P cycler
234 (Agilent). The reaction mix was prepared as follows: 1× Maxima SYBR Green mastermix (with
235 10 nM of ROX), 0.2 μM of each primer (sequences in Supplementary Table 1), 5.0 μl of DNA
236 (50 ng μl^{-1}) or 20×diluted cDNA and nuclease-free water to a final volume of 25 μl. The
237 amplification programme included an initial denaturation at 95°C for 10 min, followed by 40

cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s. A dissociation curve was performed at the end of each run. For quantification, recombinant DNA plasmid standard curves from 10^1 to 10^7 gene copies were prepared and used for quantifying the copy number from each sample as described by Adamek et al. [22].

For normalisation of expression, the gene encoding the 40S ribosomal protein S11 (*40s*) was used as reference gene [22, 25-27]. The level of gene expression is shown as the copy number of the gene normalised against 1×10^5 copies of the 40S ribosomal protein S11 (normalised copy number) using the following formula:

Normalised copy number = mRNA copies per PCR for target gene / (mRNA copies per PCR for reference gene / 10^5)

2.8. Statistical analysis

SigmaPlot 12 software (Systat Software) was used for statistical analysis. The survival rates were transformed using ArcSin. Normalised gene expression data and virus load were transformed using a Log10(x) transformation before further statistical analysis. Significant differences ($p \leq 0.05$) in virus load and gene expression during infection were assessed using a two-way ANOVA with subsequent pairwise multiple comparisons using the Holm-Sidak method. Data from primary fin cultures were evaluated using a one-way ANOVA with subsequent pairwise multiple comparisons using the Holm-Sidak method. Data are presented as box plots of 25%-75% (± minimum and maximum values) with an indication of median with Prism 7 software or as bars indicating mean (+ standard deviation) (GraphPad Software).

3. Results

3.1.Spring viremia of carp virus infection *in vivo*.

The carp strains responded to an SVCV infection with only slight differences in survival rates. The Rop strain had a 100% survival rate in all three infection experiments. The mean survival rate of both the AS and koi strain was 95% (90%, 95% and 100%, respectively in three parallel infection experiments), and 78% for the PS strain (80%, 75% and 80%, respectively). The survival rate of the PS strain was statistically significantly different when compared with the Rop strain (Fig. 1). The main onset of mortality occurred between 15 and 23 d p.i.. Kidneys collected from all fish which died were confirmed to be SVCV positive by RT-qPCR. No mortality was recorded in the control tanks. In the SVCV experiment four tissues (skin, gills, head kidney and kidney) were sampled and analysed because they play a significant role in the pathogenesis of this virus. These tissues were selected to reliably measure virus spread and immune responses: gills and skin are the portals of entry of SVCV, the kidney is one of the target tissues for the virus, while head kidney is one of the most important lymphoid organs. Normalised copy numbers of the gene encoding the G protein of SVCV were analysed in all sampled tissues of carp. This analysis revealed an immediate virus presence in the skin by 12 h p.i. and a high virus load in this tissue at the later time points in fish from all carp strains. In the gills, kidney and head kidney, the G protein encoding RNA was detected from 36 h p.i. onwards and, in particular in the kidney, the normalised copy number achieved high numbers during the further development of the infection (Fig. 2). By 96 – 336 h p.i., the SVCV G protein copy numbers in the kidney, gills and head kidney were significantly lower in the Rop strain compared to carp from the other strains at the same time points (Fig. 2). The fish from the Rop strain started to clear the infection by the last time point at 336 h p.i., while in other strains the level of the virus remained high (Fig. 2). No SVCV RNA could be detected in samples collected from mock-

infected control fish (Fig. 2).

3.2.Cyprinid herpesvirus 3 infection *in vivo*.

CyHV-3 infection of carp strains revealed high and statistically significant differences in the mean survival of fish (Fig. 1). The Rop carp had the highest mean survival of 78% (65 %, 85 % and 85 % survival rates, respectively ~~in three parallel infection experiments~~), while koi carp had the lowest mean survival rate of only 10 % in all three infection experiments. In the PS strain, on average, 35 % individuals survived the infection (35%, 25% and 45% survival rate, respectively) and in the AS strain, the average survival rate was 53 % (45%, 50% and 65%, respectively; see Fig. 1). Mortality started at day 5 p.i. and lasted until day 15 p.i. (Fig 1). Kidneys from randomly selected n=10 fish per tank which died were confirmed to be CyHV-3 positive by qPCR. No mortality was recorded in the control tanks. The same tissues like in the SVCV experiment were collected as they also play a major role in the CyHV-3 infection process. Skin is main portal of entry of CyHV-3, furthermore skin together with the gill epithelium are the most affected mucosal tissues. The kidney is one of the main target tissues for the virus, while head kidney one of the main organs where immune responses take place. Increasing virus load and CyHV-3 replication (CyHV-3 ORF72 expression) were initially observed in the skin (by 12 and 36 h p.i.) and in the gills, in kidney and head kidney later on during the course of infection (by 96 to 144 h p.i.) (Figs. 3 and 4). The tissues of koi harboured significantly higher virus loads, and a higher virus replication could be seen by 96 to 336 h p.i. compared to the tissues of Rop carp with significantly less virus and lower virus replication in all tissues considered (Figs. 3 and 4). The difference was greater in the internal tissues: Kidney and head kidney. Furthermore, at the final time point (336 h p.i.), the virus load was still rising in koi, while it was decreasing in Rop. This indicates that, at this time point, similar to the SVCV infection experiments, Rop already was able to start the process of clearing the viral infection. No CyHV-3 DNA or RNA could be

308 detected in samples collected from mock-infected fish (Figs. 3 and 4).

309

310 **3.3. Type I interferon response *in vivo***

311 During both infections the expression of the genes encoding IFN a2 (*ifn a2*) and the
312 interferon-induced protein viperin (*vig1*) were analysed. The genes encoding these two proteins
313 were selected, based on our previous experiments on type I IFN responses in common carp [16,
314 17]. The responses were measured in all four tissues in which the virus load and replication were
315 evaluated. During SVCV infection, the transcription of the *ifn a2* gene was strongly upregulated,
316 in particular by 96 and 144 h p.i. in the kidney and head kidney (Fig. 5). Likewise, the
317 transcription of the *vig1* gene was upregulated by 96 and 144 h p.i. in all examined tissues (Fig.
318 6). Overall, the activation of the type I IFN system followed a comparable pattern in all examined
319 carp strains. However, in tissues collected from Rop carp, alongside a lower virus load, lower
320 upregulation of *ifn a2* and *vig1* gene transcription was observed (Fig. 5 and Fig. 6).

321 During CyHV-3 infections a constitutive expression of *ifn a2* and *vig1* varied between
322 carp strains. Control carp from the AS strain had the highest expression level of *ifn a2*, while carp
323 from the Rop strain had the lowest one in the gills, kidney and head kidney. Control carp from the
324 PS strain had the highest expression level of *vig1* compared to other strains (Fig. 7). During the
325 infection in the skin, a slight upregulation of *ifn a2* expression was observed in fish from all
326 strains by 96 h p.i.. Carp from the Rop strain showed significantly higher upregulation of the *ifn*
327 *a2* transcription at 144h p.i. in the skin compared to fish from other strains. Interestingly, the
328 expression of *ifn a2* in the gills was downregulated at 96 h p.i. in AS and PS strains and at 336 h
329 p.i. in the AS strain. In the kidney and head kidney, the expression of *ifn a2* varied greatly in the
330 tissues of infected and non-infected carp from all strains. Furthermore, a significant regulation of
331 this gene in both tissues could not be observed in carp infected with CyHV-3. However, when the

proportional increase of *ifn a2* transcription is considered, the increase was higher in internal tissues of carp from Rop strain than in the other strains (results of "fold increase" of the transcription of this gene, estimated on the means of the transcription level are presented in Supplementary Table 2), although the absolute level of *ifn a2* expression was lower in Rop strain than in AS strain and not different to koi (Fig. 7).

The expression of *vig1* was significantly upregulated in carp from all strains infected with CyHV-3 in the kidney and head kidney of carp (from 12 to 144 h p.i. in the kidney and from 36 to 144 h p.i. in the head kidney) when compared to non-infected controls (Fig. 8). In gills, an upregulation was seen in the most susceptible koi by 96 and 144 h p.i. (Fig. 8). At the same time points, the expression of *vig1* was also upregulated in the skin of fish from all infected strains. A difference in the transcription rate of this gene between strains was noticed in non-infected control carp and in CyHV-3-infected carp at 336 h p.i.. Then expression was at its highest in most PS individuals, while Rop carp had the lowest transcription level when assessed as normalised copy number of *vig1*.

~~From these data, it can be concluded that during both *in vivo* infections, the pattern of type I IFN responses could not be responsible for the resistance of the Rop strain to both infections. Neither could this explain the higher susceptibility of koi to CyHV-3 or PS carp to SVCV infections.~~

3.4. Role of fin/skin epithelium in CyHV-3 infection *in vitro*.

In order to further analyse the role of fin/skin cells in the outcome of the CyHV-3 infection, primary cultures from fin cells were raised from carp belonging to all four genetic strains. Subsequently, these were infected with CyHV-3 in order to mimic an infection of epithelium of carp with this virus *in vitro*. The type I IFN response of the cells to the CyHV-3

infection did not differ in cell cultures raised from the fins of the different carp strains (Fig. 9). These results supported the conclusion that during CyHV-3 infection, differences in disease outcome were unrelated to differences in virus replication and antiviral interferon response in skin/fin epithelium as a primary site of infection. These results also confirmed that in the *in vitro* model, virus replication or innate immune responses would not be different in primary cell cultures derived from carp with different susceptibility to CyHV-3. Therefore, we used previously established cell lines which had proved to be susceptible to CyHV-3 infection for further *in vitro* studies.

3.5. Modulation of type I IFN responses in carp cells infected with CyHV-3 *in vitro*.

The synthetic analogues of dsRNA (poly I:C), B-form dsDNA (poly dA:dT) or of unusual Z-form ds DNA (poly dG:dC) were delivered by a cationic lipid transfection agent to KFC cells. Subsequently, interferon responses were monitored by analysing the mRNA transcription of the genes encoding the carp IFN a2, viperin, and IRF7 proteins. This analysis revealed an immediate and strong upregulation of the transcription of all measured genes upon stimulation with poly I:C, between 2h – 8 h post transfection, and to a lower extent at later time points (Fig. 10). In cell cultures stimulated with B-DNA or Z-DNA analogues, the transcription of *ifn a2*, *vig1* and *irf7* genes was upregulated as well, but at later time points, between 8h – 36h or 96 h post stimulation, respectively (Fig. 10).

In order to analyse the influence of the stimulation of an antiviral IFN type I response by synthetic ligands of the viral nucleic acid sensors, we infected stimulated and non-stimulated KFC cells with CyHV-3. In poly I:C stimulated cells infected with CyHV-3, the transcription of *ifn a2*, *vig1* and *irf7* genes was regulated in a similar pattern compared to stimulated non-infected cell cultures, with a strong upregulation by 2 and 8 h p.i,. and to a lower extent, at later time

380 points. In contrast to this, the transcription of *ifn a2* and *vig1* genes was significantly less
381 upregulated in cell cultures stimulated with B-DNA or Z-DNA and subsequently infected with
382 CyHV-3 when compared to stimulated non-infected cultures at all time points (Fig.10). In
383 addition to the transcription of interferon related genes, virus replication was analysed in the cells
384 transfected with the nucleic acid analogues as well. This analysis showed that CyHV-3
385 replication remained unaltered in cells stimulated by the synthetic analogue of Z-form dsDNA or
386 B-form dsDNA, while a transfection with poly I:C resulted in a depression of virus replication
387 (Fig. 11).
388

4. Discussion

The development of strains of animals in directed and undirected breeding programmes can influence the disease susceptibility and thus result in the development of disease-resistant strains. This can be related with a higher efficiency of immune responses to the given pathogen. Reduced disease susceptibility can also be related to a host-pathogen incompatibility; for instance, by differences in the receptor(s) required for pathogen internalisation into the cells and the successful development of an infection [28].

Magnitude of immune responses based on virus-induced interferons plays a critical role in the outcome of several virus infections in fish. This was most clearly shown for the VHSV infection in rainbow trout [13] and very recently for CaHV infection in Prussian carp [14]. Several viruses. however, have the potential to manipulate the type I IFN response to establish infection in their hosts more successfully. The present study on SVCV and CyHV-3, combined with our latest studies on CEV [18], show that in carp strains (AS, Rop, PS and koi), the differences in antivirus responses based on type I interferon are not crucial for the survival of the infected carp. Individuals from genetically different carp strains ~~greatly~~ differed in their susceptibility to the infection. Survival during SVCV infection was strain dependent and carp from the Rop strain (100% survival rate) were the most resistant, while carp from the PS strain (78% survival rate) were the most susceptible. Carp from the Rop strain had also the highest survival rate during CyHV-3 infection (78%), while only 10% of infected koi carp survived, which indicated that this strain was highly susceptible to infection with this virus. These differences in the mortality rate were also reflected in differences in virus load and in differences in virus replication. This is in line with results in other viral infections in fish. The resistance of carp from the Rop strain to CyHV-3 was shown in earlier experimental infections [6]. Nonetheless, when the results from this previous study are compared to the present findings, the

AS strain underperformed in our experiment [6]. This might be caused by a selection of less resistant parental animals because intra-strain variability was shown to play an important role in the resistance level of this strain [4, 8]. Of interest, in a previous study, we observed that AS carp from the same reproduction were most resistant to infections with CEV from genogroups I and IIa [18]. Compared to CyHV-3, significantly less is known about genetic resistance of carp to an SVCV infection. In research carried out by Kirpichnikov et al. [9], the long selection process of the SVCV resistant Krasnodar carp was presented. Interestingly, carp from the Rop strain were used as parental animals in this procedure, indicating that the improved performance of this strain in our infection experiment is not coincidental [9]. However available literature directly comparing the susceptibility of carp strains to SVCV infection in experimental conditions is very sparse. The only infection experiments in a controlled aquarium environment compared crossing between Ukrainian Ropsza (UR) and local mirror carp (L) from the Krasnodar Region in Southern Russia with “non-selected control fish” and showed differences in survival of more than 50 percentage points. This confirmed that the increased resistance had a genetic background [9].

In the current study Rop carp had significantly lower CyHV-3 and SVCV loads in all tissues but the difference was particularly high in the kidney and head kidney when compared to carp from the more susceptible strains (koi and PS). According to our initial hypothesis, this could theoretically be related to type I IFN responses, which limited the spread of the viruses to the internal tissues. However, when the type I IFN response to the CyHV-3 infection was analysed in fish from all strains, the transcription rate of genes was surpassingly low. In some cases, the type I IFN response could be correlated to the virus load. These findings disproved our initial hypothesis that the resistance of carp to CyHV-3 is based on efficient type I IFN responses. In the case of SVCV, the IFN response was much stronger; however, the same tendency could be

436 noticed. The strain with a higher virus load had a higher type I IFN response, which could
437 especially be seen in the expression of *ifn a2*.

438 It is worth mentioning that we based our conclusions on the evaluation of differences in
439 the total number of gene copies found in carp from the strains and not on a proportional increase
440 in the transcription of the gene from non-infected to infected individuals ("fold increase"). There
441 were differences in the transcription rate of IFN encoding genes in non-infected control fish from
442 the different carp lines. The mean mRNA expression level of *ifn a2* was lower in non-infected
443 Rop. Therefore, the actual fold increase in *ifn a2* mRNA copies was higher in the kidney and
444 head kidney of infected individuals when compared to carp from other strains. However, in carp
445 from this line, the absolute level of expression of this gene was lower than in AS and was not
446 different from koi.

447 The magnitude of type I IFN responses in other fish species was proven to be essential for
448 the survival of both alloherpesvirus and rhabdovirus infections. Nevertheless, this was concluded
449 from studies performed on clonal fish lines with a very low intra-strain variability [13, 14]. In our
450 studies, we had to rely on outbred strains during infection studies. However, in order to limit the
451 variability, we moved to *in vitro* conditions and used primary cell cultures derived from fish from
452 all four strains. These *in vitro* studies showed that type I IFN responses to a CyHV-3 infection
453 were similar in epithelial cell cultures from all four strains, suggesting that differences in the
454 magnitude of antiviral responses of cells between the genetic strains were not responsible for the
455 differences in disease resistance. This is in contrast to reports on salmonids, where the
456 susceptibility of individuals to infections with VHSV was correlated to the rate of virus
457 replication in fin explants and in permanent cell cultures derived from fish of these lines as well
458 as to the IFN response of these cells to the infection with the virus [13].

459 The observation that the magnitude of the type I IFN response was much lower in carp
460 infected with CyHV-3 than in carp from the same strains with an SVCV infection, led us to
461 perform another *in vitro* study which should shed some light on whether CyHV-3 would be able
462 to manipulate the type I IFN response. Viruses with a large DNA genome develop several
463 strategies which help to evade detection of a virus infection of the host cell and which provide
464 successful replication in the cells [29, 30]. In mammals, foreign (viral) DNA is recognised by
465 host cells via specific pattern recognition receptors. Many mammalian DNA sensors have
466 recently been identified, and most sensors operate via STING, a protein shown to have a central
467 role in controlling altered gene induction in response to DNA *in vivo* [31]. However, not all of the
468 sensors for virus infection (intracellular sensors of dsRNA/ viral DNA) known from higher
469 vertebrates are described in fish. In the zebrafish genome, many genes from the DNA sensing
470 cascade could be located, and in goldfish, STING serves as a distinct IFN gene activator [32]. In
471 addition, the genome of cyprinid fishes encodes a paralogue of the RNA-dependent protein
472 kinase (PKR) containing Z-DNA binding domains (PKZ), which activates antiviral IFN
473 responses in fish cells [33]. The activation of the IFN-system by poly I:C, synthetic ds RNA, was
474 previously shown in carp cells. It could be demonstrated that the activation of the IFN system by
475 poly I:C could retard the spread of a CyHV-3 infection in a cell culture [17]. In addition, in the
476 present study, the type I IFN system could be stimulated by treating cells with B-DNA and Z-
477 DNA, which are known as type I IFN activators in mammalian cells. A subsequent infection of
478 treated cells with CyHV-3 demonstrates that the upregulation of the type I IFN response by the
479 stimulators was reduced in cells under virus infection. Also, the stimulation of cells with B-DNA
480 and Z-DNA did not affect virus replication. In contrast, the upregulation of the type I IFN
481 response was faster and stronger after poly I:C stimulation, which reduced the virus replication,
482 this being similar to previously shown results [17]. These data indicate that CyHV-3 has the

potential of downregulating the type I IFN response at a very early stage of infection. These data could also suggest that CyHV-3 has the ability to avoid antiviral activities induced by cellular sensing of foreign DNA. At the current stage of research, we have not yet been able to explore this mechanism. However, recently a functional protein binding Z-form dsDNA was confirmed in the genome of CyHV-3. This protein is encoded by ORF112 [34] and is similar to the viral Z-DNA binding protein E3L in poxviruses [35]. Poxviruses use their E3L Z-DNA-binding protein to block interferon responses [35]. Therefore, a similar role was anticipated for the Z-DNA-binding protein encoded by CyHV-3 ORF112 [34]. This hypothesis might be supported by the data presented here. However, more studies are needed to confirm the cause of this phenomenon.

In the case of SVCV and CyHV-3 there are no indications that resistance was related to a complete incompatibility of the virus to the receptor used during the entry process into the host cells, providing full resistance to the pathogen, as it was proposed in the case of a VHSV resistant clonal strain of rainbow trout [13]. Likewise, in Atlantic salmon (*Salmo salar*), quantitative trait loci for *infectious pancreatic necrosis virus* (IPNV) resistance were shown to be linked with epithelial cadherin (*cdh1-1* and *cdh1-2*), most likely preventing or highly impairing virus attachment and internalisation into cells [28]. Ropsha and Amur wild carp were infected by the SVCV and CyHV-3, and the virus replicated in cells of these carp, as shown in our current study and in previous studies [8]. Importantly, these carp strains are less likely to develop the final stages of clinical KHVD which leads to the occurrence of mortality. However, infected individuals from these stains could shed infectious virus particles. Therefore, the existence of more resistant and/or tolerant strains like Rop and AS, which can be infected with the virus but are not killed by the infection, could enhance spreading of the virus. Due to the high probability of developing a latent/persistent infection, resistant strains could support viral spreading, thus inducing mortality in more susceptible individuals and populations.

In summary, our experimental infections showed surprising differences in the mortality rate of carp strains during both SVCV and CyHV-3 infections. This could be related to increased resistance to the infections leading to lower virus loads and virus replication in internal tissues of the carp from the Ropsha strain. However, the results proved our initial hypothesis wrong. In fact, the expression level of type I IFN genes was not a good marker of resistance because it was not positively correlated with survival. In the case of a CyHV-3 infection, this could be related to the potential ability of the virus to limit type I IFN responses induced by sensing viral DNA by infected host cells. Taken together, the results broaden our understanding on how common carp from different stains interact with viral pathogens.

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Figures

Fig.1. Survival of carp from the different genetic strains Amur wild carp (AS), Ropsha carp (Rop), Prerov scaly carp (PS), and koi from infections with spring viremia of carp virus (SVCV,

upper row) and cyprinid herpesvirus 3 (CyHV-3, lower row). For both viruses, the experiments were performed in three independent tanks. Different letters indicate the statistically significant difference ($p < 0.05$) in cumulative survival rate between strains for carp at given timepoint.

Fig.2. Spring viremia of carp virus (SVCV)-infection in carp from the genetic strains Amur wild carp (AS), Ropsha carp (Rop), Prerov scaly carp (PS), and koi. Depicted are normalised copy numbers of (i) mRNA transcripts encoding the SVCV specific G protein as surrogate for virus replication, skin, gills, kidney and head kidney at various time points post infection. Different letters indicate the statistically significant difference ($p < 0.05$) between strains for carp at given timepoint. Data are presented as box plots of 25%-75% (± minimum and maximum values) with an indication of median as a horizontal line.

Fig.3. Cyprinid herpesvirus 3-infection in carp from the genetic strains Amur wild carp (AS), Ropsha carp (Rop), Prerov scaly carp (PS), and koi. Depicted are normalised copy numbers of (i) CyHV-3 specific DNA as surrogate for virus load in skin, gills, kidney and head kidney at various time points post infection. Different letters indicate the statistically significant difference ($p < 0.05$) between strains for carp at given timepoint. Data are presented as box plots of 25%-75% (± minimum and maximum values) with an indication of median as a horizontal line.

Fig.4. Cyprinid herpesvirus 3-infection in carp from the genetic strains Amur wild carp (AS), Ropsha carp (Rop), Prerov scaly carp (PS), and koi. Depicted are normalised copy numbers of mRNA transcripts encoding the CyHV-3 specific gene ORF72 as surrogate for virus replication in skin, gills, kidney and head kidney at various time points post infection. Different letters indicate the statistically significant difference ($p < 0.05$) between strains for carp at given

555 timepoint. Data are presented as box plots of 25%-75% (± minimum and maximum values) with
556 an indication of median as a horizontal line.
557
558 **Fig.5.** Spring viremia of carp virus (SVCV)-infection in carp from the genetic strains Amur wild
559 carp (AS), Ropsha carp (Rop), Prerov scaly carp (PS), and koi. Depicted are normalised copy
560 numbers of mRNA transcripts encoding ifn a2 as indicator for antiviral interferon responses of
561 carp in skin, gills, kidney and head kidney at various time points post infection. * indicates
562 statistically significant difference ($p < 0.05$) to the control, different letters indicate the
563 statistically significant difference ($p< 0.05$) between strains for carp at given timepoint. Data are
564 presented as box plots of 25%-75% (± minimum and maximum values) with an indication of
565 median as a horizontal line.
566
567 **Fig.6.** Spring viremia of carp virus (SVCV)-infection in carp from the genetic strains Amur wild
568 carp (AS), Ropsha carp (Rop), Prerov scaly carp (PS), and koi. Depicted are normalised copy
569 numbers of mRNA transcripts encoding vig 1 as indicator for antiviral interferon responses of
570 carp in skin, gills, kidney and head kidney at various time points post infection. * indicates
571 statistically significant difference ($p < 0.05$) to the control, different letters indicate the
572 statistically significant difference ($p < 0.05$) between strains for carp at given timepoint. Data are
573 presented as box plots of 25%-75% (± minimum and maximum values) with an indication of
574 median as a horizontal line.
575
576 **Fig.7.** Cyprinid herpesvirus 3-infection in carp from the genetic strains Amur wild carp (AS),
577 Ropsha carp (Rop), Prerov scaly carp (PS), and koi. Depicted are normalised copy numbers of
578 mRNA transcripts encoding ifn a2 as indicator for antiviral interferon responses of carp in skin,

gills, kidney and head kidney at various time points post infection. * indicates statistically significant difference ($p < 0.05$) to the control, different letters indicate the statistically significant difference ($p < 0.05$) between strains for carp at given timepoint. Data are presented as box plots of 25%-75% (± minimum and maximum values) with an indication of median as a horizontal line.

Fig.8. Cyprinid herpesvirus 3-infection in carp from the genetic strains Amur wild carp (AS), Ropsha carp (Rop), Prerov scaly carp (PS), and koi. Depicted are normalised copy numbers of mRNA transcripts encoding vig1 as indicator for antiviral interferon responses of carp in skin, gills, kidney and head kidney at various time points post infection. * indicates statistically significant difference ($p < 0.05$) to the control, different letters indicate the statistically significant difference ($p < 0.05$) between strains for carp at given timepoint. Data are presented as box plots of 25%-75% (± minimum and maximum values) with an indication of median as a horizontal line.

Fig.9. Primary cell cultures of fin derived cells of carp from the genetic lines Amur wild carp (AS), Ropsha carp (Rop), Prerov scaly carp (PS), and koi. Left panel: transcription of CyHV-3 OFR 72 as surrogate of virus replication after CyHV-3 infection of the cells, central panel: transcription of the gene encoding ifn a2, and, right panel: transcription of the gene encoding vig 1 as indicators for an induction of antiviral IFN responses. * indicates statistically significant difference ($p < 0.05$) to the control, different letters indicate the statistically significant difference ($p < 0.05$) between strains for carp at given timepoint. Data are presented as mean +SD.

Fig.10. Stimulation of carp cells by synthetic analogues to B-form dsDNA (Poly dA:dT), Z-form

603 ds DNA (Poly dG:dC), and dsRNA (Poly I:C) as ligands to cytosolic sensors of DNA or dsRNA,
604 and infection of stimulated cells with CyHV-3. Effect on mRNA expression encoding for type I
605 IFN (ifn a2) and the IFN stimulated gene *vig 1* at 2h, 8h, 36h and 96h post stimulation/ infection.
606 * indicates statistically significant difference ($p < 0.05$) to the control, # indicates the statistically
607 significant difference ($p < 0.05$) between CyHV-3 infected and non-infected cultures at given
608 timepoint. Data are presented as mean +SD.
609
610 **Fig.11**. Stimulation of carp cells by synthetic analogues to B-form dsDNA, Z-form ds DNA, and
611 dsRNA (Poly I:C) as ligand to cytosolic sensors of DNA or dsRNA, and infection of stimulated
612 cells with CyHV-3. Effect on the transcription of the virus specific gene encoding ORF 55 as
613 surrogate for virus replication. # indicates the statistically significant difference ($p < 0.05$)
614 between CyHV-3 infected and treated cell cultures at given timepoint. Data are presented as
615 mean +SD.
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619 **References**

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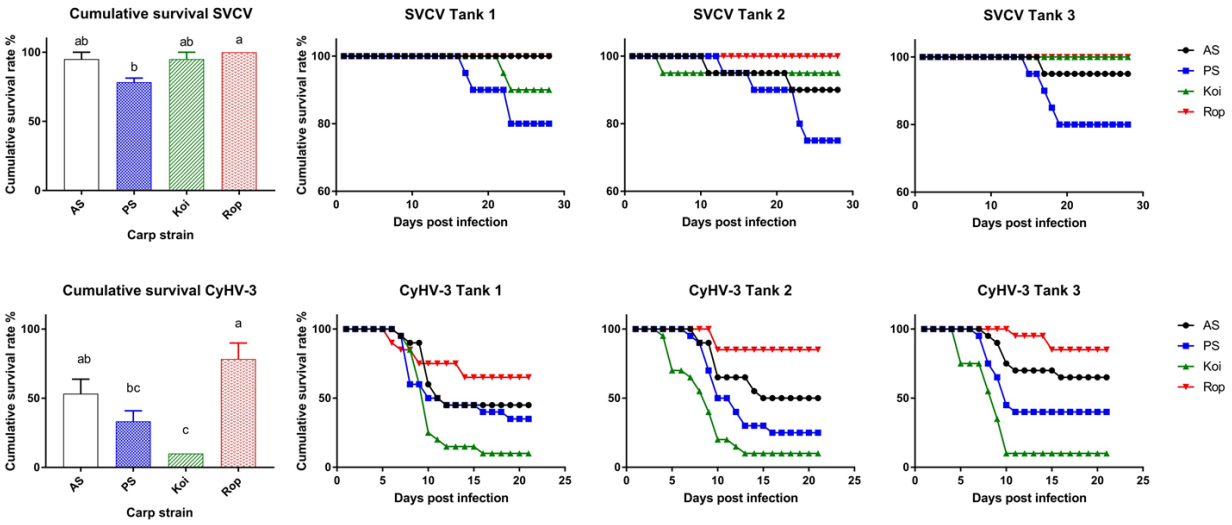


2h
ifn a2

8h
ifn a2

36h
ifn a2

96h
ifn a2

vig1

vig1

vig1

vig1

irf7

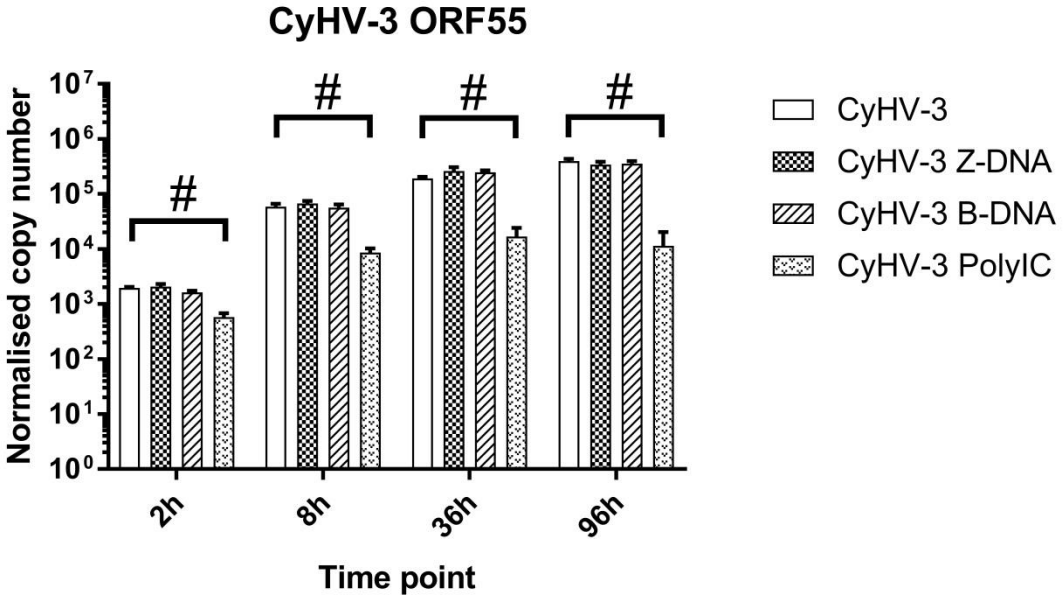
irf7

irf7

irf7

Normalised copy number

Control
CyHV-3
Z-DNA
CyHV-3 Z-DNA
B-DNA
CyHV-3 B-DNA
PolyIC
CyHV-3 PolyIC


CyHV-3 ORF55
Normalised copy number
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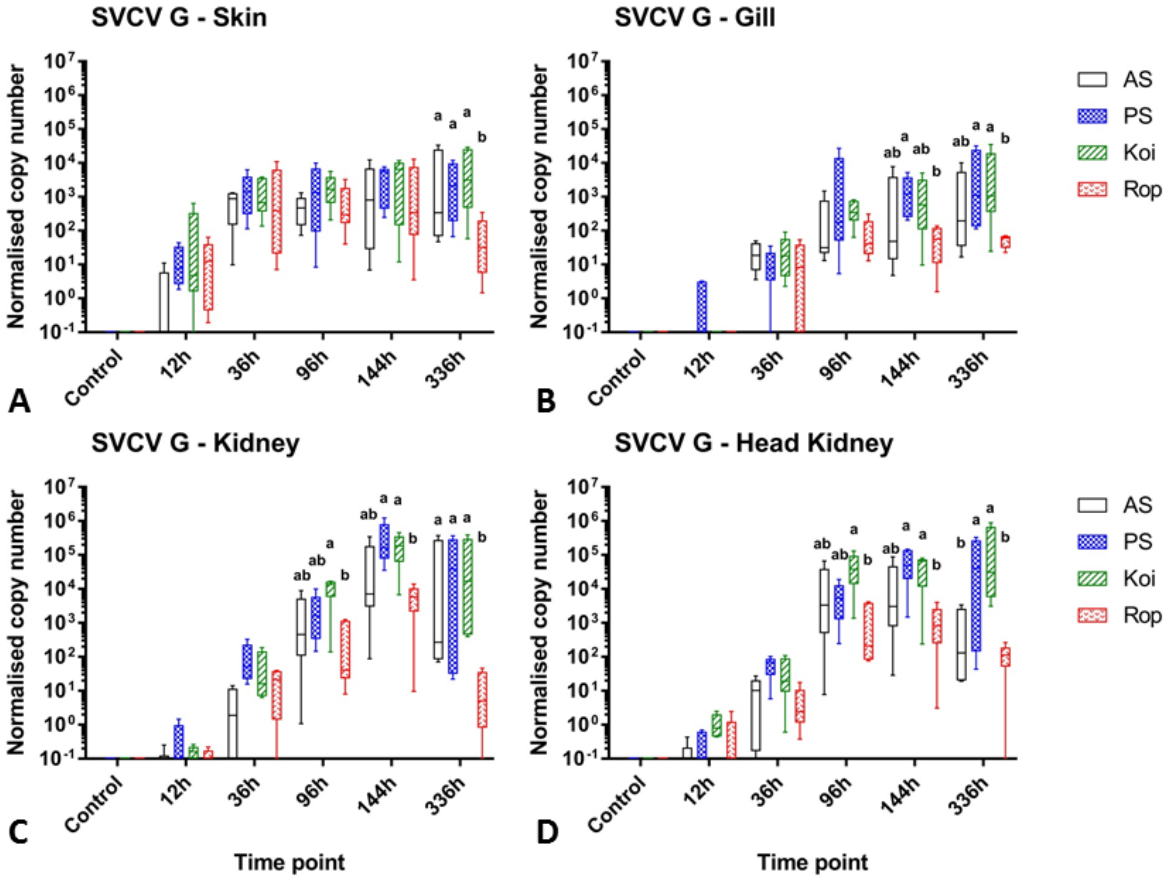
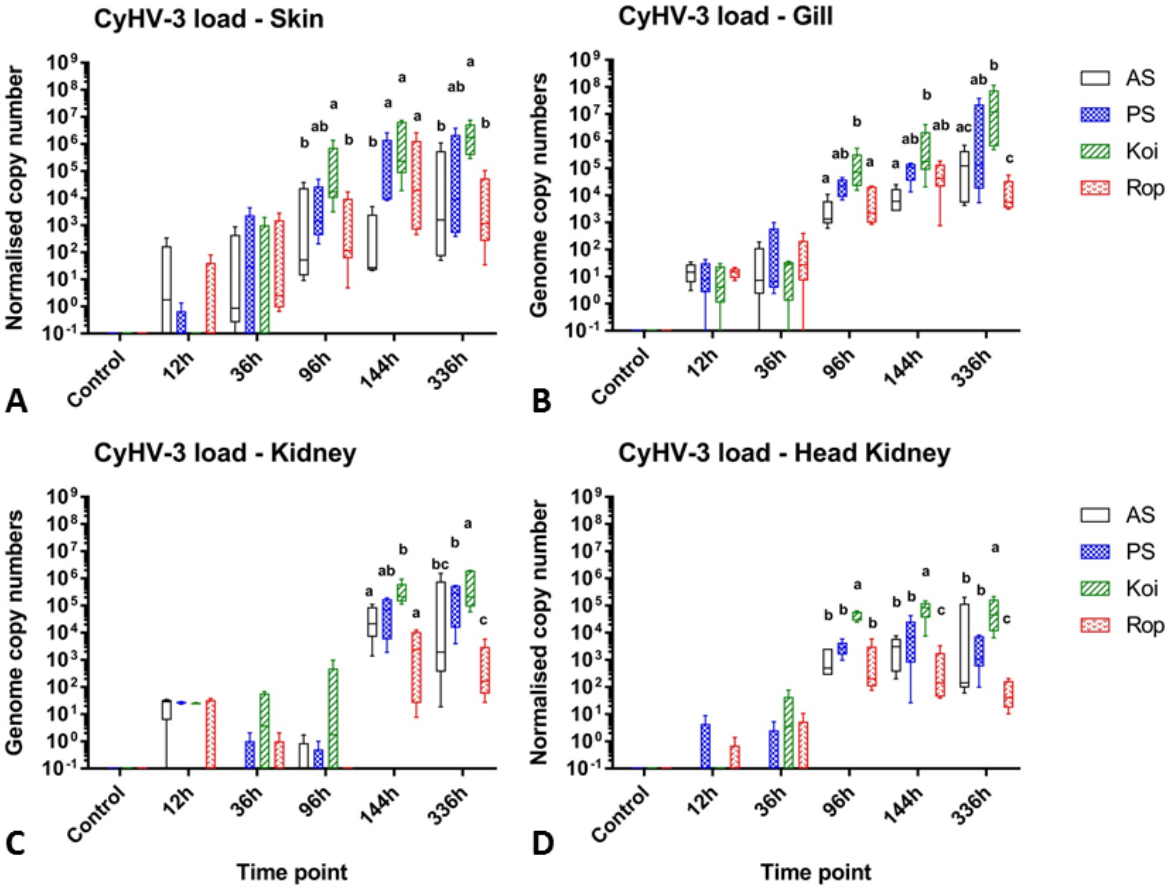
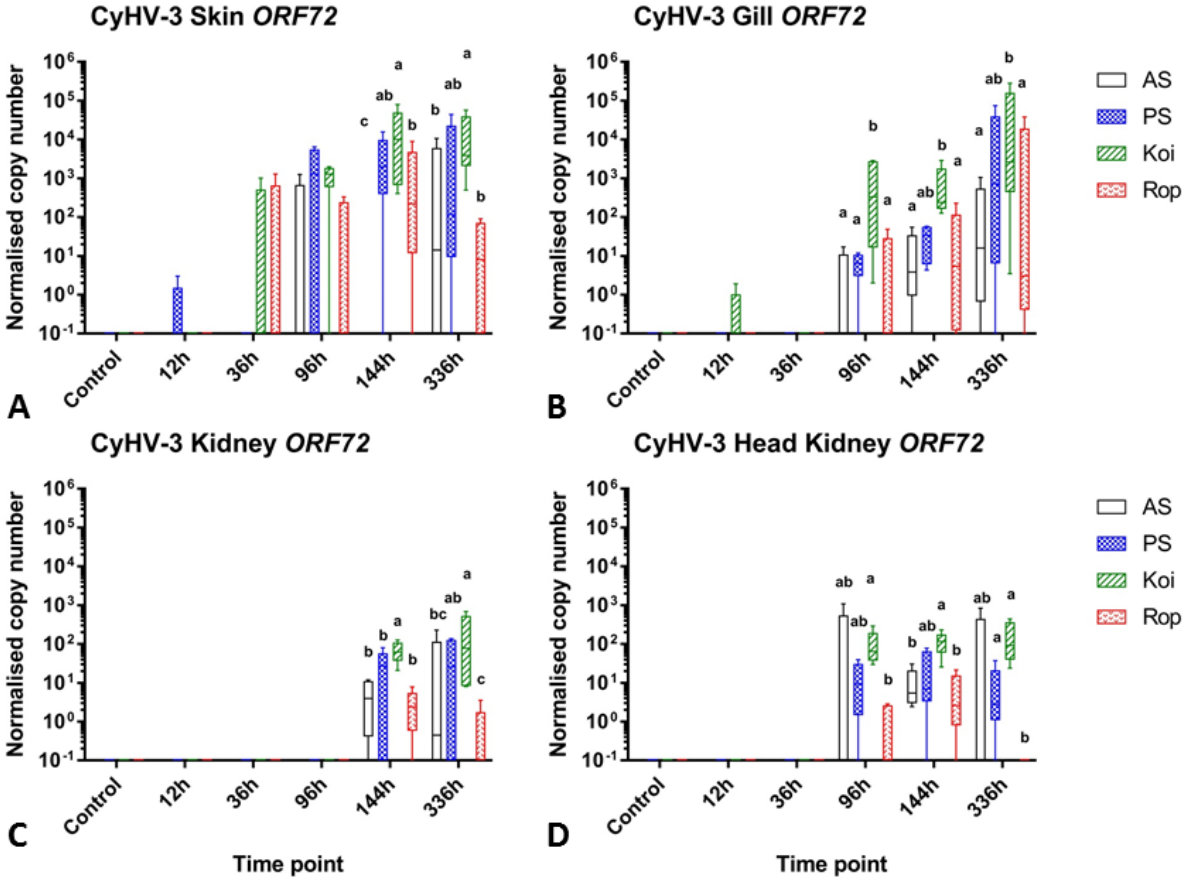
10^6
10^5
10^4
10^3
10^2
10^1
10^0
#
#
#
#
2h
8h
36h
96h
Time point
CyHV-3
CyHV-3 Z-DNA
CyHV-3 B-DNA
CyHV-3 PolyIC


SVCV G - Skin
SVCV G - Gill
SVCV G - Kidney
SVCV G - Head Kidney
Normalised copy number
Control
12h
36h
96h
144h
336h
Time point
AS
PS
Koi
Rop
A
B
C
D




CyHV-3 Skin ORF72
CyHV-3 Gill ORF72
CyHV-3 Kidney ORF72
CyHV-3 Head Kidney ORF72
Normalised copy number
Control
12h
36h
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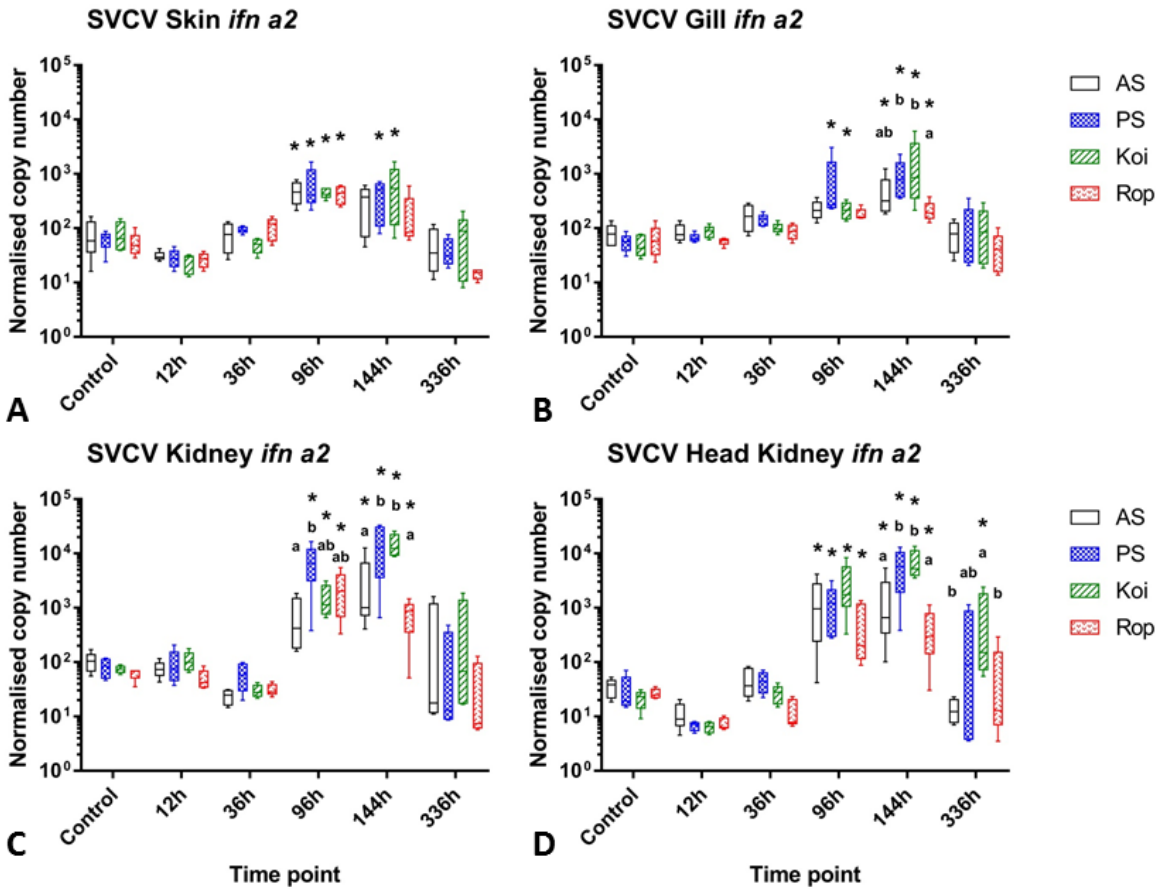
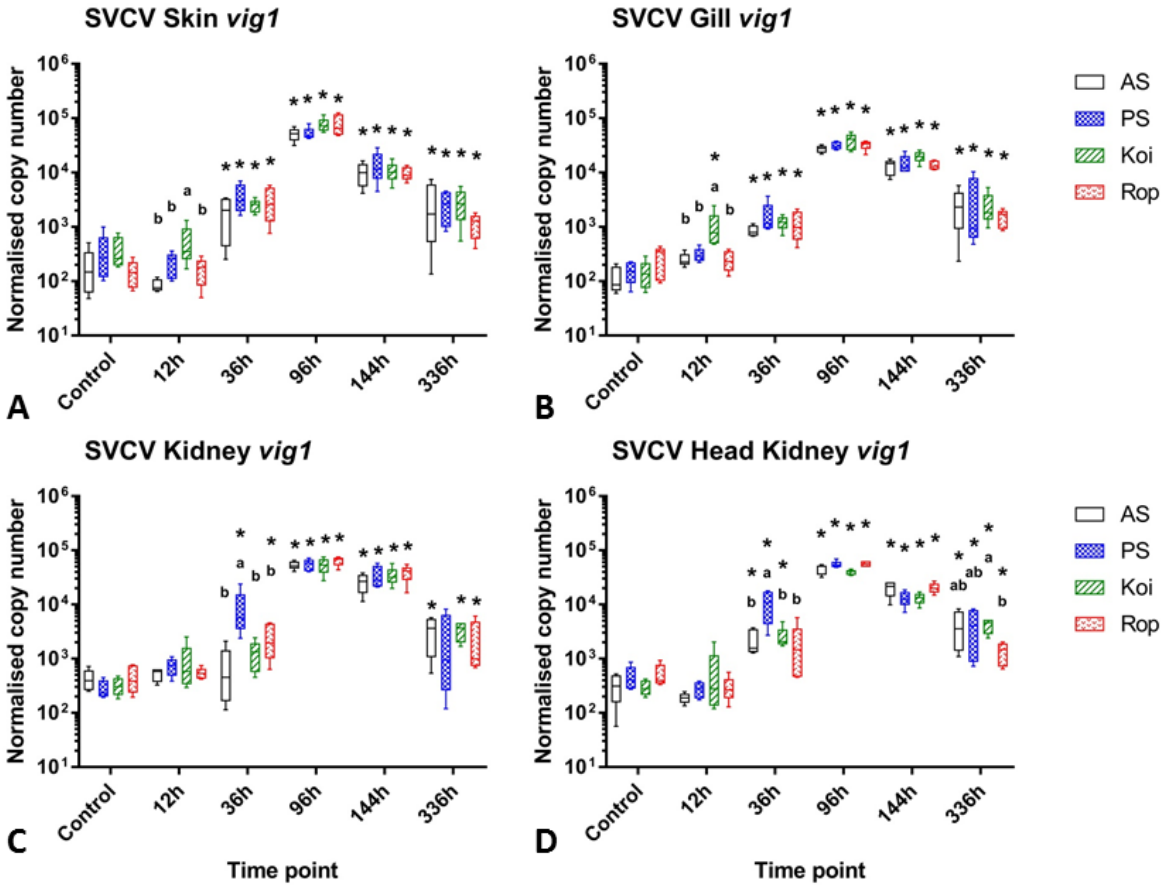
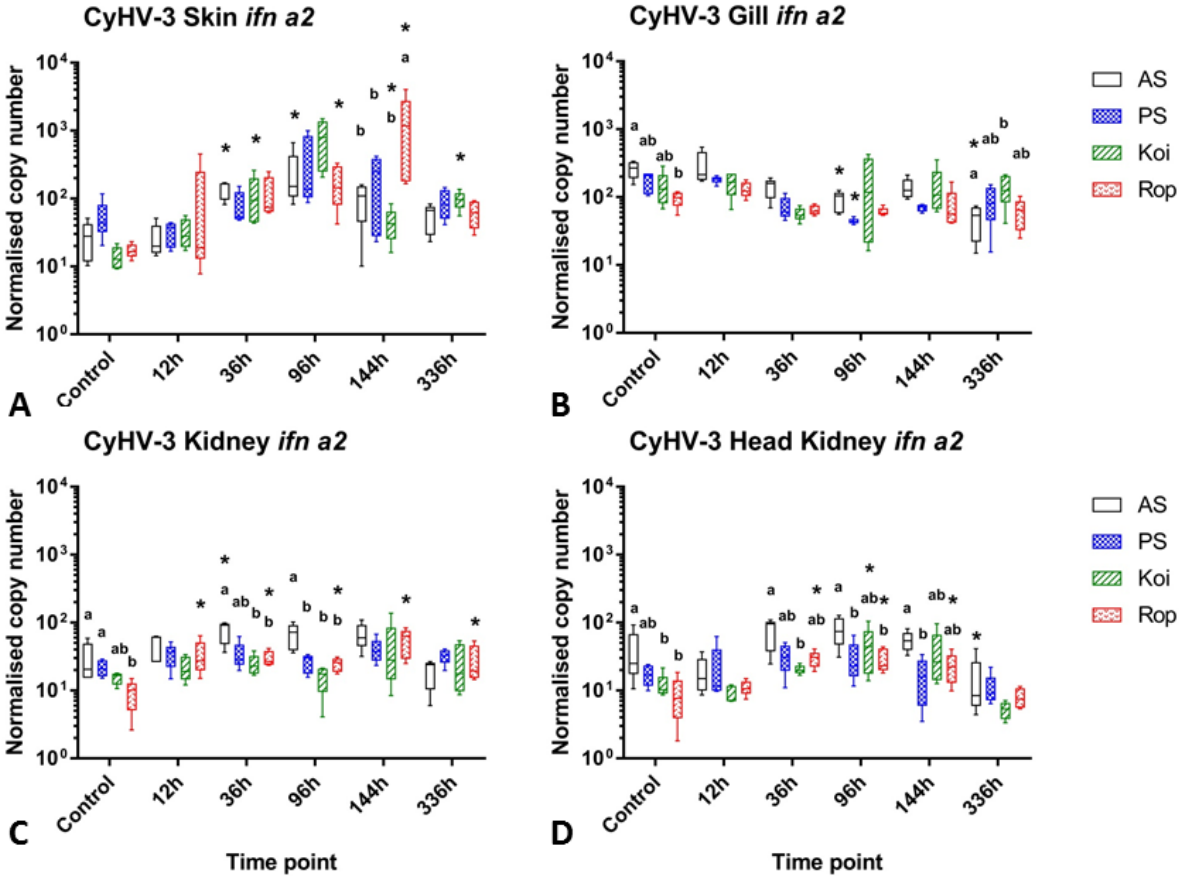
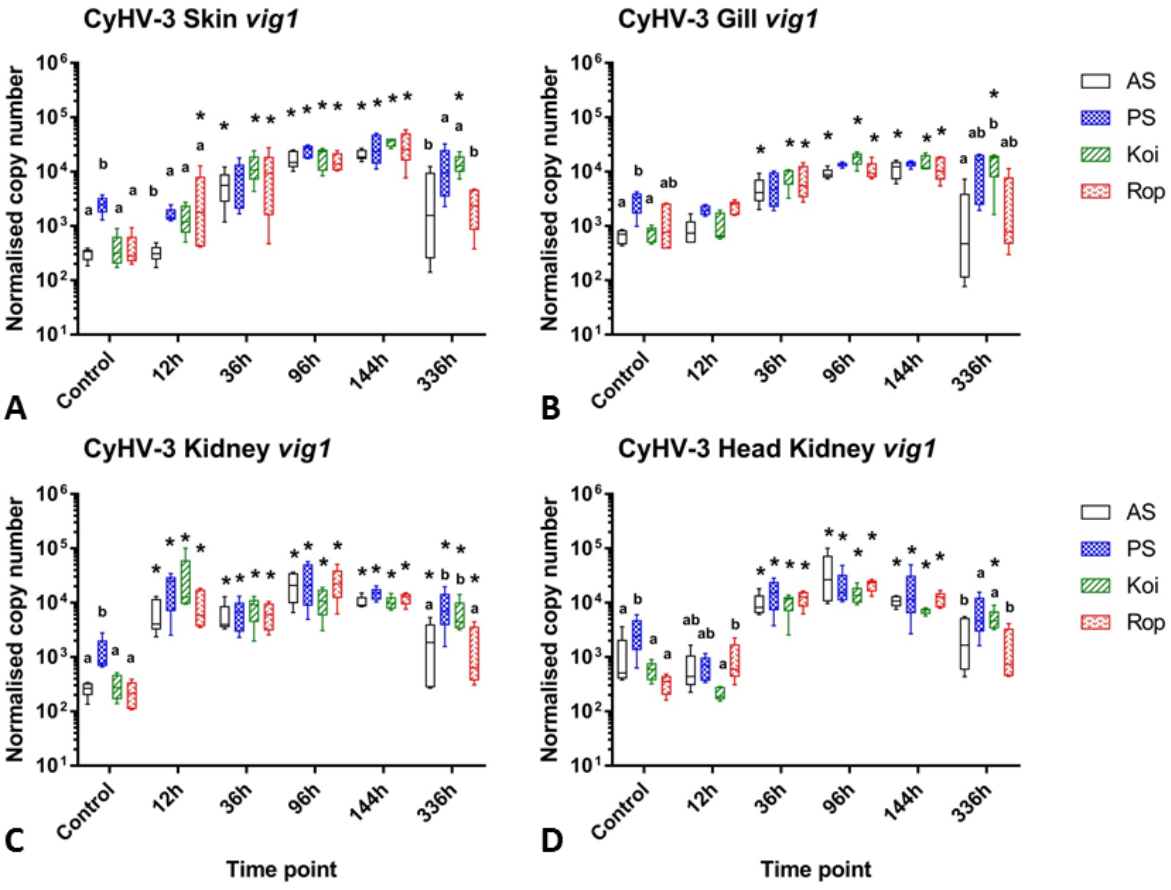
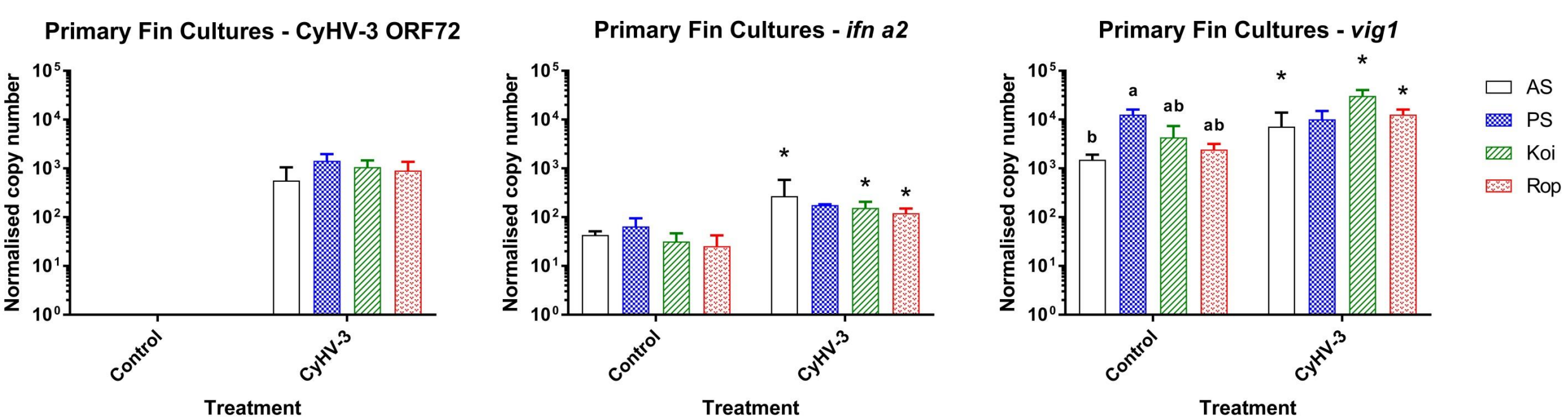
144h
336h
Time point
AS
PS
Koi
Rop
A
B
C
D




SVCV Skin vig1
SVCV Gill vig1
SVCV Kidney vig1
SVCV Head Kidney vig1
Normalised copy number
Control
12h
36h
96h
144h
336h
Time point
A
B
C
D
AS
PS
Koi
Rop


CyHV-3 Skin ifn a2
CyHV-3 Gill ifn a2
CyHV-3 Kidney ifn a2
CyHV-3 Head Kidney ifn a2
Normalised copy number
Control
12h
36h
96h
144h
336h
Time point
AS
PS
Koi
Rop
A
B
C
D




Primary Fin Cultures - CyHV-3 ORF72
Normalised copy number
10^5
10^4
10^3
10^2
10^1
10^0
Control
CyHV-3
Treatment
Primary Fin Cultures - *ifn a2*
Normalised copy number
*
*
*
Control
CyHV-3
Treatment
Primary Fin Cultures - *vig1*
Normalised copy number
b
a
ab
ab
*
*
*
Control
CyHV-3
Treatment
AS
PS
Koi
Rop

Highlights

1. Differences in mortality rates during SVCV and CyHV-3 infections were recorded in carp strains.
2. The higher resistance of the Rop strain was related to lower virus load and replication.
3. The magnitude of type I IFN response was not positively correlated with survival.
4. CyHV-3 has an ability to limit IFN response induced by sensing viral DNA by cells.